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PROCEEDINGS

OF THE

INDIAN ACADEMY OF SCIENCES

VOL. XXXVII] *SECTION B* [No. 4

APRIL 1953

Price Rs. 2 or 3 Sh. *Annual Subscription Rs. 18*

PRINTED AT THE BANGALORE PRESS, BANGALORE CITY, BY C. VASUDEVA RAO,
SUPERINTENDENT (I/C), AND PUBLISHED BY THE INDIAN ACADEMY OF
SCIENCES, BANGALORE.

CARBON, NITROGEN AND pH RELATIONSHIPS TO GROWTH OF SOIL FUSARIA IN CULTURE*

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Received January 23, 1953

(Communicated by Prof. T. S. Sadasivan, F.A.Sc.)

Introduction

Among plant pathologists and plant physiologists, there is to-day, perhaps, no topic of more engaging interest and no problem more difficult to solve than that of the nature of causal agency in wilt diseases in plants. Methods available for limiting the virulence of the pathogens include the application of ' trace elements ' to the wilt-sick soils which boosts up the bacterial population (Sadasivan, 1951), certain strains of which are known to destroy the pathogen by producing some toxins, or amending the soils with various nitrogenous fertilizers (Leach and Davey, 1942; Smith and Walker, 1941; Dick and Tisdale, 1938; Dick, 1939; and Sadasivan, 1952). As far as the latter statement is concerned reports of Fulton (1927), Kulkarni (1934), Pincard and Leonard (1944) are contradictory to the observations of Fahmy (1927) and Dastur (1931) who showed that application of organic fertilizers had adverse effects on the growth of the plant in the presence of pathogen or in other words enhanced wilt. Such contradictory results obtained on the application of nitrogenous fertilizers in combating wilt diseases led the author to investigate the problem " Nitrogen metabolism of pathogenic fungi " as a collaborative study in the University Botany Laboratory and Madras Christian College Chemical Laboratory.

Pure culture studies of the pathogenic fungi *Fusarium vasinfectum* and *Fusarium moniliforme* which are responsible for the wilt of cotton and paddy respectively, were made using different sources of nitrogen, since for conclusive investigations of any micro-organism, pure culture studies are not only desirable but also absolutely essential. Further a controlled study which permits reproducible results always depends on the use of pure cultures.

While the author recognises the importance of studying the response of host-parasite to nitrogen amendment, the present study was confined to investigations on the effect of nitrogen on the pathogens alone in pure culture;

* Part of thesis approved for the Degree of Master of Science of the University of Madras.

this would enable one to understand more thoroughly the host-parasite relationships. Moreover, the importance of the problem "Nitrogen Metabolism of Pathogenic Fungi" becomes all the more obvious, when we consider that the disease reaction in plants is the resultant of host-parasite metabolic activities and to understand clearly the 'wilt' concept in higher plants, a thorough study of the physiology of the parasite is of primary importance and basic prerequisite.

MATERIALS AND METHODS

The general technique followed for preparation of media, and maintenance of cultures was according to Rawlins (1933). Special methods used are detailed below.

The organisms were grown on 100 ml. of Richard's nutrient medium (Rawlins, 1933) contained in 250 ml. Pyrex flasks, the nitrogen source being varied to give four different kinds of media, potassium nitrate, ammonium sulphate, urea and preptone, forming the different sources of nitrogen. Each nitrogen source was then supplied at four different levels of 28 mg., 70 mg., 140 mg. and 210 mg. in 100 ml. of medium. Chemicals used were ANALAR grade (B.D.H.).

pH adjustments were effected with N/10 HCl or N/10 NaOH making use of Beckman electronic pH meter. pH was adjusted to 5·5 prior to autoclaving.

Sterilization of media was conducted at 10 lb. steam pressure for 5 minutes.

Method of inoculation.—Seven-day old cultures of the fungi growing on potato dextrose agar in Pyrex glass test-tubes formed the source of inoculum. Bits of aerial mycelium were used for inoculation because of increasing evidence of essentiality of 'trace elements' and vitamins and growth-promoting factors (Wolf and Wolf, 1947), which are likely to be present in the agar that is used for the purpose. All the flasks were incubated at the laboratory temperature of 28–31° C. At the intervals shown in Text-Figs. 1 to 4, three flasks from each level were taken out for analyses, determination being made for (1) dry weight of the fungal mat; (2) pH of filtrate; (3) total nitrogen content of fungal crop; (4) unconsumed nitrogen (*a*) total, (*b*) NO_3–N, and (*c*) NH_4–N; and (5) reducing sugars in the filtrate.

Dry weight of fungal mat.—Fungal mats were removed by filtration using previously weighed Whatman 42 filter-papers and dried to constant weight at 45° C. as advocated by Ping and Allison (1944), due correction being made for the weight of filter-paper.

The filtrate obtained was concentrated to 80 ml. over an electric heater at 70° C. and hydrolysed with 4 ml. of concentrated hydrochloric acid for 20 minutes at the same temperature. It was then made up to 100 ml. and reserved for nitrogen and sugar analyses.

Reducing sugars were determined according to a method devised by the author which is a modification of Cole's (1933) alkaline ferricyanide method and has been described elsewhere (Pai, 1952).

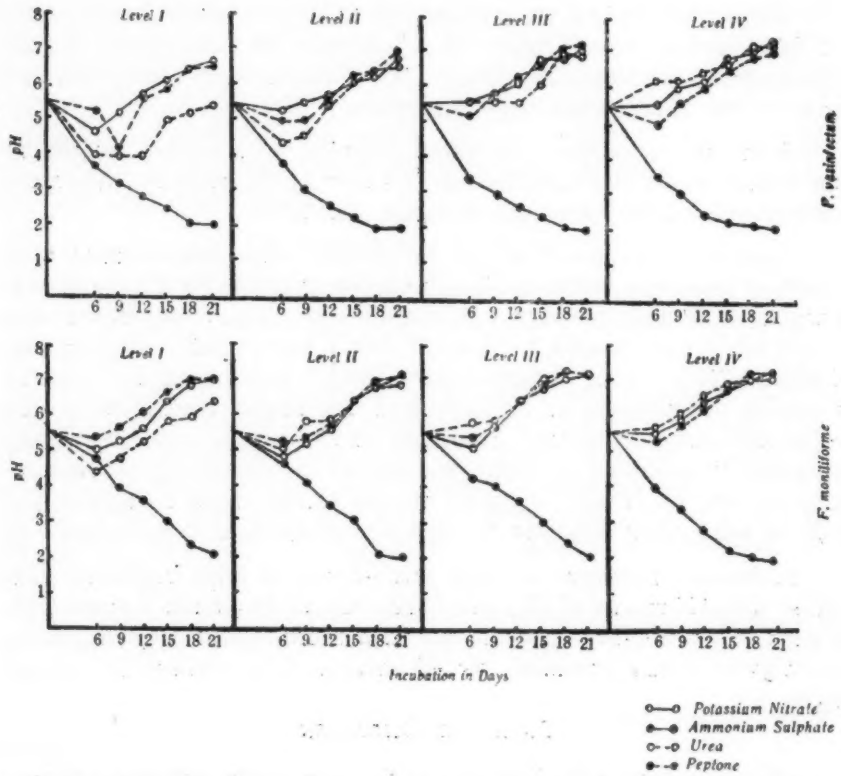
Ammonia nitrogen.—10 ml. of the filtrate were steam-distilled with 10 ml. of phosphate buffer of Nichols and Foote (1931) for 4 minutes in a Parnas and Wagner apparatus. Nitrate nitrogen: 2 ml. of the filtrate were boiled for 10 minutes with 25 ml. of 5% NaOH and cooled. This was then distilled with 0·2 g. of Devardas alloy. In the above cases the liberated ammonia was collected in 2% boric acid and titrated against N/100 HCl using MaZuazaga indicator. Estimation of nitrogen in filtrate containing considerable quantities of sugar was carried out according to Anantakrishnan and Pai (1952). If the filtrate was rich in nitrate nitrogen it was reduced using dilute sulphuric acid and iron filings prior to kjeldahlization.

Estimation of nitrogen in fungal mat.—50 mg. of dried fungal mat were mixed with 100 mg. of K_2SO_4 and 50 mg. $CuSO_4.5H_2O$ and digested with 2·5 ml. of nitrogen-free sulphuric acid in a micro-kjeldahl digestion flask, the digestion being continued for 60 minutes after clarification of the digest.

Data and Discussion

pH relationships.—The highlights of the experimental evidences accumulated in the present study is discussed in relation to work already carried out along similar lines, so as to obtain a clearer insight into the metabolic activities of the pathogenic fungi *Fusarium vasinfectum* and *Fusarium moniliforme* which have been observed to be physiologically alike (Sadasivan, 1951).

In the present study it was found that the reaction of the media in which the fungi grew, was influenced by the form and source of nitrogen metabolized by them (Text-Fig. 1). With both fungi, when KNO_3 formed the source of nitrogen, the tendency was to lower the hydrogen-ion concentration, thereby raising the exponent pH towards alkalinity, but during the entire period of incubation hydrogen-ion concentration in *Fusarium vasinfectum* series was higher than in *F. moniliforme* series, irrespective of the levels of nitrogen used. The amount of nitrate nitrogen present in the medium also played an important part in the shift of pH. The lowest level showed a higher concentration of hydrogen-ions than the immediately

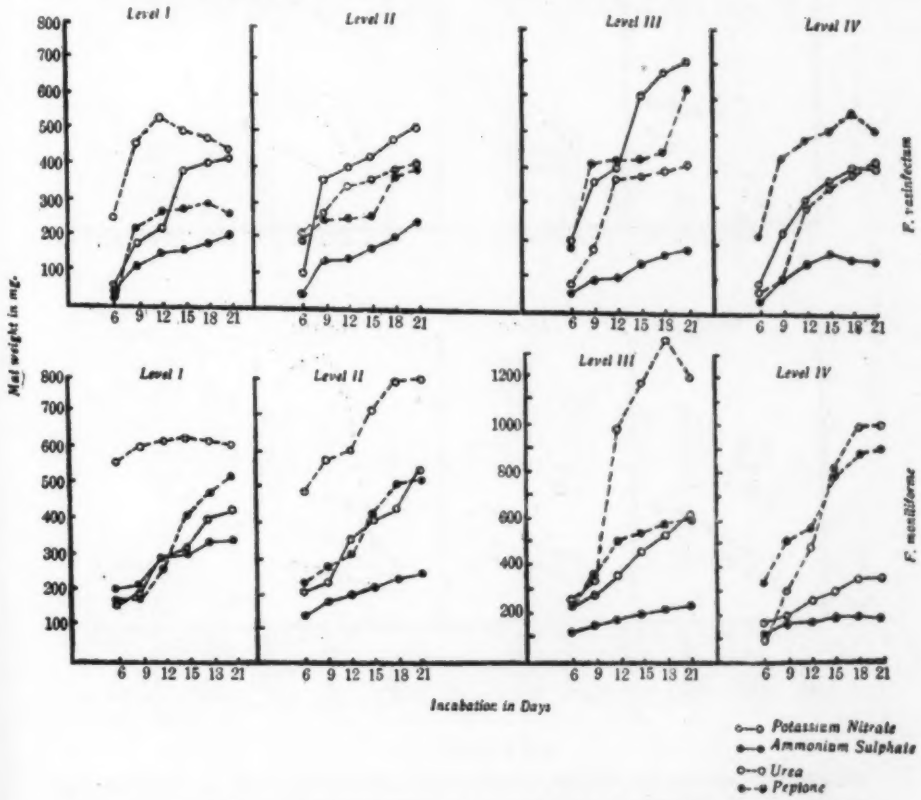


TEXT-FIG. 1. pH of substrate with the growth of *F. vasinfectum* and *F. moniliforme* with different sources of nitrogen at different levels.

higher one in the early stages of incubation, although on prolonged incubation reaction at all levels tended towards neutrality. Ramakrishnan (1941) in his investigations on *Colletotrichum* showed that whatever may be the initial pH value of the nutrient medium (he used Richard's nutrient medium with KNO_3 as a nitrogen source) the final values (after the growth of the fungus) were always in the region of neutrality.

When ammonium sulphate formed the source of nitrogen, pH dropped progressively with period of incubation with both fungi at all concentrations of the salt used. As in the case of KNO_3 series, *F. vasinfectum* showed a higher hydrogen-ion concentration than *F. moniliforme*. Further, with both fungi increasing the concentration of the salt resulted in increasing the hydrogen-ion concentration on prolonged incubation. After three weeks fungal growth pH of the substrate was about 2·0–2·1.

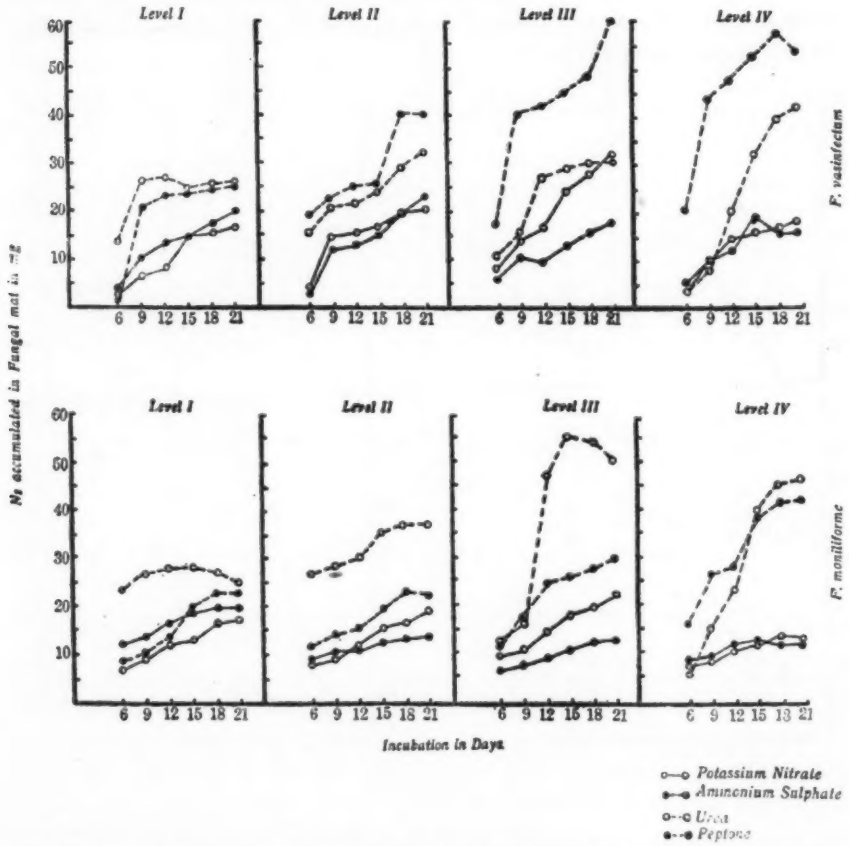
First two levels in urea series exhibited pronounced lowering of pH with both Fusaria and these on prolonged incubation rose towards neutrality. With higher levels, pH rose steadily on incubation. In levels I and II, in the early stages of fungal growth, both species were seen to be very active as judged by the mat weights obtained and the sugar consumed (Text-Figs. 2 and 4). The fall in pH may, in all probability, be due to the rapid formation of organic acids, resulting from sugar metabolism (Pratt, 1924) and the utilization of these acids as carbon sources (Moore, 1924) subsequently with the consequent increase in pH.



TEXT-FIG. 2. Dry weight of mycelial mat of *F. vasinfectum* and *F. monilliforme* with different sources of nitrogen at different levels.

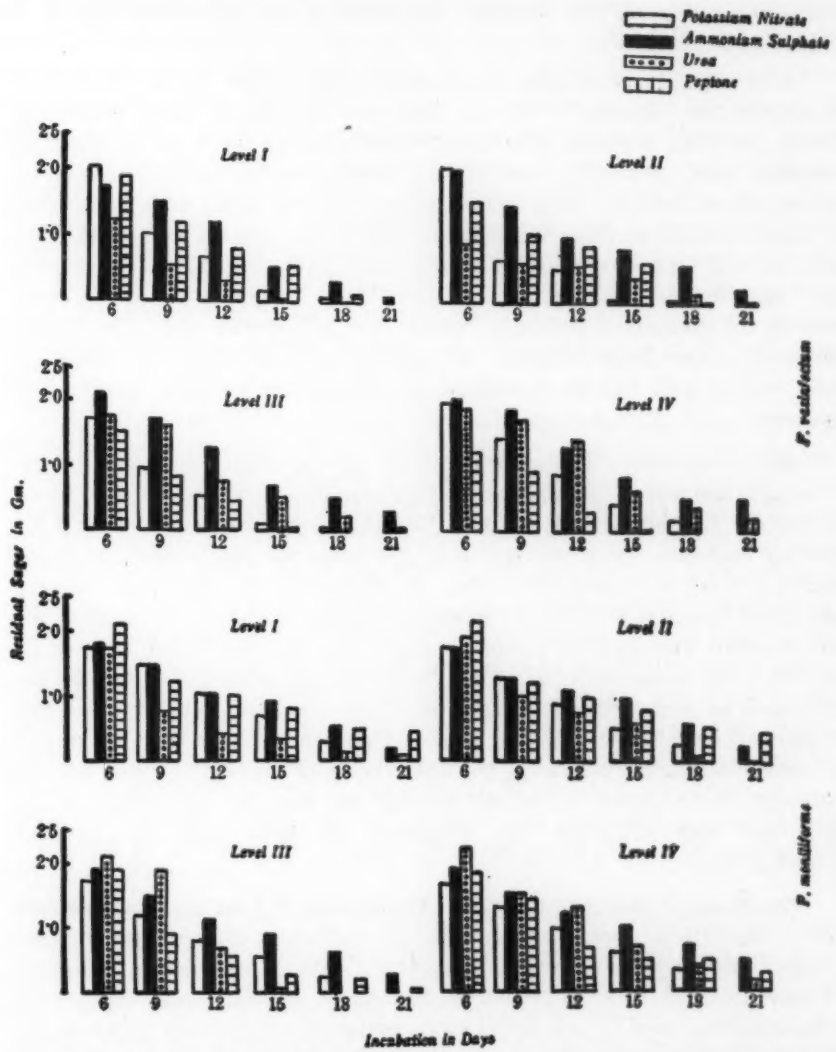
A small drop in pH values in peptone series with both fungi in the early stages of incubation observed in the present investigation agrees with the findings of Klotz (1923) with *Aspergillus niger*, *Sphæropsis malorum* and

Diplodia natalensis. He concluded that organic acids formed during the decomposition of sugar were responsible for the increased hydrogen-ion concentration in the early stage of incubation, and the decrease in the hydrogen-ion concentration was brought about by the removal of these acids for the building up of fungal tissues on prolonged incubation, when carbohydrates were also completely depleted.



TEXT-FIG. 3. Amount of nitrogen accumalated in fungal tissue of *F. vasinfectum* and *F. moniliforme* with different sources of nitrogen at different levels.

Waksman (1920) in his researches on the metabolism of Actinomycetes concluded, that $NaNO_3$ containing nutrient medium became alkaline after the growth of the organism; when ammonium salts of strong acids were added to the media, the reaction became acidic. Boyle (1924) obtained



TEXT-FIG. 4. Residual sugar in substrate of *F. vasinfectum* and *F. moniliforme* with different sources of nitrogen at different levels.

an alkaline reaction with Fusaria grown in basic Richard's nutrient medium. Earlier Nikitinsky (1904) working on the physiology of soil fungi had observed that when NH_4Cl was used as a nitrogen source in nutrient medium it became acidic due to the removal of NH_3 and accumulation of HCl in

substrate; when peptone formed the source of nitrogen, alkalinity set in with growth of fungi.

In a separate experiment conducted by the author it was found that *F. vasinfectum* continued to live for well over 225 days in media containing KNO_3, $NaNO_3$, peptone, urea (which produced ammonia as an autolytic product) and $(NH_4)_2SO_4$ and NH_4Cl (which became intensely acidic on prolonged incubation). This behaviour is quite interesting when considered alongside with the findings of Zachariah (1949) who isolated the fungus from air-dried soil after two years of storage and Kulkarni (1934) who showed that the fungus could remain viable in fields for well over twelve years even in the absence of the host, cotton. It is probable that the fungus survived on the easily available nitrogenous and carbonaceous matter in soils and in pure culture it utilized the carbon and nitrogen stored up in its tissue.

Mat production.—Taking mat weight as an index—with both fungi *F. vasinfectum* and *F. moniliforme*, the physiological similarity in *plus* KNO_3 media was very remarkable in that they grew very luxuriously at level III (140 mg. nitrogen %) of the salt used, the order for mat production being level I < level II < level III > level IV (Text-Fig. 2). Increased concentration of NO_3—N at level IV decreased vegetative growth although it did not interfere with sucrose utilization (Text-Fig. 4). Decreased mat weight in $(NH_4)_2SO_4$ series, with both fungi, was inversely proportional to the concentration of salt used. The fungi were not, however, killed by the high acidity (pH 2·1) of the substrate, since the growth increased with the period of incubation thereby indicating the adaptability of the fungi to high acidity. Increased concentration of the salt lowered not only the mat weight of the fungi but also decreased the utilization of sugar from the medium (Text-Fig. 4).

Physiological dissimilarities of the two species of Fusaria, *F. vasinfectum* and *F. moniliforme* were obvious from the analytical data for mat weights obtained with organic sources of nitrogen. With *F. vasinfectum* increased concentrations of urea decreased production of mycelial mat but with *F. moniliforme*, level III was found to be optimum immediately followed by level IV. With peptone as a nitrogen source, optimum level for *F. vasinfectum* was III. *F. moniliforme* on the other hand grew more profusely with increased concentrations of peptone nitrogen. Moreover, mat weights obtained with urea and peptone for *F. moniliforme*, were higher than for *F. vasinfectum* thus showing the adaptability of the former to organic sources of nitrogen to a greater extent.

Sugar utilization.—With both fungi, rates of depletion of sugar were directly proportional to the mat weight obtained, the actual rates being governed by the nitrogen source (Text-Fig. 4). When sugar in the medium was exhausted, either the growth of Fusaria slackened or there was a positive decrease in mat weight. In some cases as in urea level I, growth of fungi was inhibited because of complete utilization of nitrogen from substrate (Text-Fig. 2 and Tables I and II). Slackening of growth rate observed in the case of KNO_3 series coincides with the complete depletion of sugar (Text-Figs. 1 and 4). With organic sources of nitrogen like peptone although the growth was slackened with complete depletion of sugar it was not pronounced, thereby indicating that the Fusaria utilized the non-nitrogenous carbon residue of the peptone molecule for the building up of their mycelial mat.

In urea series at level I, sugar depletion was complete after 18 days incubation with *F. vasinfectum*, but with higher concentrations of nitrogen, considerable amounts of sugar were still left behind in the substrate. This anomaly could not be due to the utilization of carbon that was present in urea molecule in preference to the readily available sugar, for, if that were the case, the mat weight should have increased with increasing concentrations of urea. But it was observed that the mat weight actually decreased on increased concentration of the nitrogen source (Text-Fig. 2). It may be that higher concentrations of urea are not conducive to the growth of the pathogen. A similar observation was presented by Cochrane and Conn (1950) with *Streptomyces cæliocolor* which gave a negligible amount of vegetative growth and utilized less than half the amount of glucose supplied with an incubation period of 8 days in the presence of urea. *F. moniliforme* simulated *F. vasinfectum* in that the rate of depletion of sugar was proportional to the mat produced, yet differing in that urea levels III and IV were better than I or II (Text-Fig. 2).

With peptone, however, physiological activities exhibited by both Fusaria were similar, since both used considerable amount of sugar as a source of carbon in the presence of sufficient peptone equivalent to 70, 140 and 210 mg. N_2%. It is probable that the pathogens have to break up the peptone molecule before gaining an access to the nitrogenous portion to achieve which, the energy is obtained from the sugar contained in the medium. Parallel to this Boas (1919) pointed out that nitrogen in the form of amino acids was not very easily utilizable by micro-organisms; much energy was needed for the process of deamination which was obtained from the sugars supplied. This was further corroborated by Klotz (1923) who obtained very good growth of *Aspergillus niger*, *Diploidia natalensis* and *Sphæropsis malorum*

TABLE I

Showing the Residual Nitrogen in the Substrate of Fusarium vasinfectum

RESIDUAL NITROGEN

Nitrogen source	Incubation in days	Level I			Level II			Level III			Level IV		
		Total	NO_3-N	NH_4-N	Total	NO_3-N	NH_4-N	Total	NO_3-N	NH_4-N	Total	NO_3-N	NH_4-N
Potassium nitrate ..	6	23·6	21·24	..	61·42	59·86	..	122·5	120·2	..	198·6	197·4	..
	9	18·92	17·14	..	57·25	55·42	Traces	118·5	116·1	..	192·0	189·2	..
	12	17·83	15·32	..	49·32	45·36	2·78	113·9	110·46	Traces	186·3	182·4	Traces
	15	10·44	8·59	1·4	45·84	42·46	2·84	108·8	105·34	1·4	185·7	183·2	2·42
	18	8·72	5·86	2·2	45·32	40·54	4·58	106·6	101·48	2·48	183·9	179·0	3·15
	21	8·25	4·14	3·6	44·46	37·32	6·48	102·5	100·2	2·86	183·3	175·0	4·23
Ammonium sulphate ..	6	22·81	..	21·56	65·04	..	64·48	132·7	..	129·4	204·9	..	204·84
	9	16·59	..	15·84	57·08	..	56·54	129·3	..	128·8	198·0	..	197·2
	12	12·63	..	11·28	53·98	..	52·84	130·0	..	128·4	125·6	..	193·84
	15	11·6	..	9·96	54·2	..	51·32	126·2	..	125·2	191·1	..	190·43
	18	9·9	..	8·84	49·61	..	46·46	122·6	..	120·1	191·3	..	188·26
	21	9·05	..	8·94	45·5	..	44·94	120·2	..	118·2	191·9	..	189·48
Urea ..	6	13·1	..	..	54·6	..	Traces	128·9	..	Traces	206·4	..	..
	9	4·52	..	..	49·73	..	,,	124·2	..	,,	202·2	..	..
	12	Traces	..	Traces	47·96	..	5·43	112·4	..	2·46	188·7	..	2·86
	15	,,	..	,,	45·2	..	6·46	111·3	..	3·2	175·1	..	3·2
	18	,,	..	,,	40·08	..	5·84	109·5	..	3·84	169·8	..	3·98
	21	1·86	..	1·86	31·4	..	5·12	108·9	..	5·94	168·5	..	5·98
Peptone ..	6	25·54	..	Traces	49·54	..	..	121·9	..	..	188·8	..	..
	9	7·87	..	6·56	46·14	..	Traces	98·69	..	..	178·6	..	..
	12	3·81	..	3·45	43·83	..	3·84	98·27	..	Traces	162·1	..	3·84
	15	2·96	..	2·12	43·0	..	4·62	97·12	..	3·86	156·0	..	4·32
	18	2·85	..	1·98	28·66	..	5·84	92·39	..	5·98	152·8	..	5·86
	21	3·1	..	2·96	28·57	..	8·46	78·38	..	6·74	156·1	..	7·32

TABLE II

Showing the Residual Nitrogen in the Substrate of Fusarium moniliforme

RESIDUAL NITROGEN

Nitrogen Source	Incubation in days	Level I			Level II			Level III			Level IV		
		Total	NO_3-N	NH_4-N	Total	NO_3-N	NH_4-N	Total	NO_3-N	NH_4-N	Totals	NO_3-N	NH_4-N
Potassium nitrate ..	6	21·63	20·48	..	61·2	59·36	..	129·1	127·3	..	201·98	197·3	..
	9	19·4	18·94	..	60·3	59·48	..	128·5	126·48	..	200·5	197·46	..
	12	15·59	15·14	..	57·2	55·32	..	124·7	121·4	..	198·11	195·32	Traces
	15	15·04	13·43	Traces	53·36	50·14	Traces	120·9	118·14	Traces	197·1	195·46	,,
	18	11·17	9·34	,,	52·12	50·24	,,	117·9	114·84	..	194·1	191·32	,,
	21	10·64	8·26	,,	49·05	45·32	2·4	114·2	112·1	3·1	193·1	191·4	3·4
Ammonium sulphate ..	6	15·8	..	15·3	61·32	..	60·88	131·61	..	130·4	201·29	..	199·4
	9	14·86	..	13·46	59·2	..	59·14	130·58	..	128·84	198·79	..	197·84
	12	11·6	..	10·88	58·85	..	57·18	129·41	..	127·16	197·72	..	195·32
	15	9·81	..	8·96	57·79	..	55·32	128·54	..	127·2	197·85	..	196·4
	18	9·02	..	8·98	56·0	..	54·4	126·45	..	125·84	197·04	..	194·86
	21	8·98	..	7·56	55·47	..	54·88	125·61	..	123·15	196·91	..	192·34
Urea ..	6	3·7	..	..	42·66	..	..	126·05	..	..	201·99	..	..
	9	1·46	..	..	41·53	..	..	121·93	..	..	192·84	..	..
	12	Traces	..	..	39·25	..	Traces	89·15	..	Traces	184·71	..	..
	15	,,	..	Traces	34·13	..	2·86	82·91	..	4·84	168·67	..	Tra c
	18	,,	..	,,	31·55	..	5·42	83·51	..	6·38	161·99	..	4·86
	21	,,	..	,,	30·16	..	7·88	85·7	..	9·46	160·6	..	8·48
Peptone ..	6	19·71	..	..	58·03	..	..	126·69	..	..	191·94	..	..
	9	18·33	..	..	55·61	..	..	120·51	..	..	182·57	..	..
	12	15·64	..	..	53·85	..	..	115·1	..	Traces	181·18	..	2·46
	15	7·21	..	1·84	48·78	..	3·4	113·27	..	3·4	169·43	..	3·4
	18	4·01	..	1·98	45·71	..	5·48	112·14	..	6·9	166·5	..	8·62
	21	2·62	..	2·16	44·9	..	7·32	109·01	..	11·32	165·9	..	11·84

on peptone media with added glucose; growth on peptone media without glucose was meagre.

The inhibition of mat production on the complete exhaustion of available sugar and nitrogen with *F. vasinfectum* and *F. moniliforme* may be considered to fit in the statement of Klotz (1923) ".... since the carbohydrate carbon supply has been exhausted, the organism must draw upon the peptone and probably upon their own substance for both C and N to continue their metabolic activities....".

Nitrogen content of the mat.—The determination of total nitrogen in mats of Fusaria revealed the fact that more nitrogen was found in cultures grown in organic sources of nitrogen than in inorganic (Text-Fig. 3). In urea level I, almost the entire quantity of nitrogen from substrate was utilized and stored up in the fungal mycelium and with KNO_3 and $(NH_4)_2SO_4$

TABLE III

Showing the Percentage Content of Nitrogen in Tissue of Fusarium vasinfectum

	PERCENTAGE NITROGEN CONTENT OF MAT			
Nitrogen source	Incubation in days	Level I	Level II	Level III	Level IV
Potassium nitrate ..	6	4·717	4·053	3·82	4·023
	9	4·01	4·022	3·744	4·052
	12	3·778	4·047	3·858	4·393
	15	4·023	4·039	3·941	4·042
	18	3·962	4·055	4·048	3·988
	21	3·911	4·005	4·507	4·04
Ammonium sulphate	6	11·02	9·743	9·607	9·847
	9	9·683	9·858	11·06	9·107
	12	9·79	9·687	9·133	7·969
	15	9·928	9·743	9·086	9·984
	18	9·847	9·883	9·417	9·495
	21	9·328	9·665	9·543	10·14
Urea ..	6	5·328	6·966	17·16	5·032
	9	5·815	7·502	7·995	7·084
	12	5·273	5·965	7·103	6·668
	15	5·285	6·558	7·357	8·869
	18	5·525	7·228	7·353	9·458
	21	5·903	7·642	7·447	9·701
Peptone ..	6	10·16	9·731	9·692	9·038
	9	9·69	8·876	9·548	9·586
	12	9·283	10·43	9·579	9·679
	15	9·012	10·21	10·09	10·1
	18	8·351	10·28	10·27	9·847
	21	9·423	9·888	9·723	10·00

at the same level only about 50–60% of the nitrogen supplied was stored up in the fungal tissues. Moreover, synchronous with the fall in value for accumulated nitrogen in urea level I series, traces of ammoniacal nitrogen were detected in the substrate and this was due to autolysis of the fungus since there was a fall in mat weight also (Text-Fig. 2). Decrease in the nitrogen accumulated in the fungal tissue in urea level III was observed in *F. moniliforme* and this coincides with complete utilization of sugar supplied (Text-Figs. 3 and 4). With other sources of nitrogen, accumulation of the same in fungal mycelium increased with the period of incubation, this being pronounced in peptone series. Increase in the concentration of ammonium sulphate in the nutrient medium decreased the accumulation of nitrogen in fungal mat and with KNO_3 this increase in nitrogen accumulation was perceptible only upto level III after which there was a decrease. With urea, increase in the nitrogen source stimulated accumulation of nitrogen in cul-

TABLE IV

Showing the Percentage of Nitrogen in Tissue of Fusarium moniliforme

		PERCENTAGE NITROGEN CONTENT OF MAT			
Nitrogen source	Incubation in days	Level I	Level II	Level III	Level IV
Potassium nitrate	6	4·617	4·031	4·15	4·273
	9	4·507	3·947	4·00	4·388
	12	4·109	3·528	3·98	3·954
	15	3·890	3·913	3·916	3·812
	18	4·033	3·763	3·842	3·897
	21	3·904	3·644	3·784	3·814
Ammonium sulphate	6	5·996	6·068	6·145	6·024
	9	5·96	6·246	5·907	6·109
	12	5·636	5·685	5·573	6·124
	15	5·817	5·678	6·039	5·957
	18	5·692	5·809	5·670	5·941
	21	5·586	5·631	5·853	5·593
Urea	6	4·264	4·956	5·002	5·728
	9	4·357	4·952	4·972	4·121
	12	4·443	4·994	4·841	4·86
	15	4·351	5·062	4·764	4·875
	18	4·185	4·773	4·071	4·625
	21	4·08	4·771	4·150	4·448
Peptone	6	3·868	5·228	5·318	5·093
	9	5·097	5·031	4·982	5·046
	12	5·007	5·105	4·922	4·908
	15	4·918	4·908	4·85	4·844
	18	4·807	4·620	4·684	4·779
	21	4·505	4·430	4·756	4·679

tures of *F. vasinfectum*, but level III was found to be optimum for *F. moniliforme*, and an exactly reverse reaction was observed in peptone series (level III was optimum for *F. vasinfectum* and accumulation of nitrogen was proportional to the peptone content with *F. moniliforme*).

Percentage nitrogen content of the fungal mat calculated on its dry weight basis showed its decrease on prolonged incubation with *F. moniliforme*: in other words young cultures had a higher percentage of nitrogen content than older ones (Table IV). Highest percentage was obtained in $(NH_4)_2SO_4$ series which turned acidic due to fungal growth. This was followed by organic sources of nitrogen and KNO_3. Percentage nitrogen content remained almost the same for a particular source of nitrogen, which signifies the fact that, for an organism the nitrogenous portion of its tissue remains the same for a particular source of nitrogen irrespective of its concentration. Similar tendencies were observed with *F. vasinfectum* using KNO_3, $(NH_4)_2SO_4$ and peptone as nitrogen sources (Table III). With urea, however, percentage nitrogen content in mat increased with increasing concentrations of the nitrogen source. *F. vasinfectum* accumulated a higher percentage of nitrogen in its mat than *F. moniliforme* with $(NH_4)_2SO_4$, urea and peptone as nitrogen sources. With KNO_3, however, both fungi had similar figures for percentage nitrogen content.

In general it was found that the media which were made strongly acidic with the growth of the Fusaria, produced mats having highest nitrogen percentage and with *F. moniliforme* percentage nitrogen content decreased with age of the culture. Experimental results of Stokes and Gunness (1946) showed that prior to sporulation, the fungal mycelium contained more proteins, and individual amino-acids found in mats varied with the environmental conditions such as the media and age of culture. Investigations with *F. vasinfectum* and *F. moniliforme* in the present study, not only agree with Stokes and Gunness conclusions but also prove that the percentage nitrogen found in the organism does not vary considerably for a particular source of nitrogen irrespective of the concentration used, urea being an exception in the case of *F. vasinfectum*.

Residual nitrogen.—Analytical data for residual nitrogen show that complete utilization of nitrogen took place only in urea level I and peptone level I; this was not observed in the case of inorganic sources of nitrogen (Tables I and II), thus bringing out the superiority of organic sources of nitrogen in the building up of fungal tissues. This was further substantiated by the mat weights obtained for both fungi (Text-Fig. 2). Increase in total residual nitrogen was noticed in urea level III with *F. moniliforme* on

the 21st day (Table II), urea level I, peptone level I and IV (Table I), with *F. vasinfectum* and this increase was in the form of ammoniacal nitrogen. Synchronous with the increase in the residual nitrogen of the substrate, a drop in nitrogen accumulated in the fungal tissue was also noticed. Further this increase in residual nitrogen was noticed only after complete exhaustion of sugar which shows that the ammoniacal nitrogen was an autolytic product. Such increase in nitrogen content of the substrate due to autolysis of fungal tissues has also been observed by Klotz (1923). Further, in the present study, it was found that autolysis was more pronounced in the case of *F. moniliforme* which grew faster than *F. vasinfectum*.

Although there was a marked increase in the ammoniacal content coinciding with the complete exhaustion of sugar from media presence of ammoniacal nitrogen in the early stages of incubation with urea, peptone and to a lesser extent with KNO_3 as sources of nitrogen was not overlooked (Tables I and II). It was also observed that with the faster growing fungus, *F. moniliforme*, ammonia nitrogen produced was more than with *F. vasinfectum*. This substantiates the assumption of Waksman (1918) that a definite amount of ammonia was produced from materials as a waste product independent of the presence of available carbohydrates. There are no conclusive data in the literature to show that nitrates must be reduced to nitrites or ammonia before utilization although presence of nitrites during the metabolism of nitrates has been observed (Nord and Mull, 1945).

On the basis of the experimental results detailed above, it is concluded that fungi in general must be regarded as differing among themselves fundamentally in their metabolic potentialities as far as the assimilation and utilization of nitrogen and carbon is concerned. Further, it is hoped that a similar study conducted on host material which is being done in the University Botany Laboratory would not only bring about a definite correlation with the selective pathogenic relationship of the fungi to their particular hosts of choice but also elucidate to a certain extent the importance of carbon nitrogen metabolism of the pathogens as one of the deciding factors in the complex mechanism of wilt diseases in plants.

Summary

The reaction of the medium with the growing of *F. vasinfectum* and *F. moniliforme* cultures was influenced by the form and source of nitrogen metabolised by them. The hydrogen-ion concentration of media inoculated with *F. vasinfectum* was, however, higher than in media inoculated with *F. moniliforme* at all levels of nitrogen supplied. With the exception of $(NH_4)_2SO_4$ series, the others, *viz.*, KNO_3, urea and peptone series, brought

about a decrease in pH of the media during the initial 6 to 9 days growth of the fungi and subsequently increased the pH towards alkalinity or neutrality. In the case of $(NH_4)_2SO_4$ series this lowering of pH persisted throughout. Both Fusaria grew at a considerably low pH of 2·1.

Amount of mycelial mat produced during the entire period of incubation was higher in *F. moniliforme* cultures than *F. vasinfectum* cultures with all sources of nitrogen employed. Both Fusaria made best growth in media containing KNO_3 at 140 mg. nitrogen % level. Mat weights in ammonium sulphate series were inversely proportional to the concentration of the salt. In *F. moniliforme*, however, it was directly proportional upto 140 mg. nitrogen % level. Increase in concentration of peptone as a source of nitrogen in culture media (upto level 140 mg. nitrogen %) progressively increased mat weight of *F. vasinfectum* cultures; this increase in growth was directly proportional to the concentration of the nitrogen source used in the case of *F. moniliforme* series.

Percentage nitrogen in fungal mat of the two Fusaria was dependent on the age of the culture and source of nitrogen supplied and differed in the two species. Accumulation of nitrogen in the growing fungal mat was more when organic sources of nitrogen were supplied to the substratum, than when inorganic sources were supplied. Almost the entire quantity of nitrogen supplied to the substratum in the form of urea and peptone at 28 mg. % concentration was stored up in the fungal tissue of both Fusaria. This was, however, not the case when inorganic sources of nitrogen were supplied.

Sugar in culture media, containing different concentrations of KNO_3 as source of nitrogen, was depleted completely by 21 days' growth of both Fusaria. In media containing ammonium sulphate as a source of nitrogen, considerable amount of sugar was detected even after 21 days incubation of the Fusaria. No direct correlation existed between the rates of sugar depletion by the Fusaria when peptone and urea formed the sources of nitrogen. Ammonia formed in the substrate from media containing nitrate and organic nitrogen sources was the product of partial autolysis of the fungus.

The general conclusions are that both Fusaria displayed markedly different physiological relationships; this was brought out by their rates of growth, by their nitrogen and sugar utilization and by the varying nature and extent of hydrogen-ion change of the media caused by them.

ACKNOWLEDGEMENTS

The author takes great pleasure in acknowledging his gratitude and thankfulness to Professor T. S. Sadasivan, M.Sc., Ph.D. (Lond.), Director,

University Botany Laboratory, Madras, for suggesting the problem and rendering unstinted help throughout the course of the investigation and in the preparation of this paper. He is greatly indebted to Professor S. V. Anantakrishnan, M.A., Ph.D. (Lond.), Professor of Chemistry, Madras Christian College, Tambaram, for his constant guidance and helpful criticisms. Thanks are also due to the Government of India for the award of a Junior Fellowship during the tenure of which a part of the investigation was carried out.

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RELATION OF NITROGEN TO GROWTH AND SPORULATION OF *FUSARIUM VASINFECTUM* ATK.

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(Received January 23, 1953)

(Communicated by Prof. T. S. Sadasivan, F.A.SC.)

The role of nitrogen in the physiology of fungi has received considerable attention from mycologists in the past (*see* Lilly and Barnett, 1951; Hawker, 1950) and the present communication is but an addition to the voluminous literature already extant. The work reported here deals with some aspects of a study of a vascular wilt-producing species of *Fusarium*, *viz.*, *F. vasinfectum* Atk., the fungus causing cotton wilt. It is admitted that plant diseases, and especially those of a soil-borne nature, require for their elucidation study of physiology of the host, the parasite or the pathogen, and the host-parasite relationship itself (Brown, Brooks and Bawden, 1948). Comparatively little has been attempted in the investigation of such problems in relation to cotton wilt. The effect of trace elements on the physiology of *F. vasinfectum* and some other species of *Fusarium* has recently been studied in this laboratory (Yogeswari, 1948; Sadasivan, 1951). Doubtless, major elements like N, P and K are equally important. The results of a preliminary investigation on the effect of four different nitrogen sources on the growth, sporulation, nitrogen accumulation, etc., of *F. vasinfectum* are presented in this paper.

Materials and Methods

A culture of *Fusarium vasinfectum* Atk. obtained from the Government Mycologist, Coimbatore, was used. The identity of the fungus and also its pathogenicity on cotton plants have been confirmed by one of us (C. V. S.) after careful study.

For chemical analyses, the fungus was grown in 100 ml. of Richard's synthetic liquid medium (Rawlins, 1933, p. 85) in 250 ml. Pyrex Erlenmeyer flasks (three replicates each), the nitrogen source and the amount of nitrogen supplied being varied. Four different sources of nitrogen, *viz.*, potassium

nitrate, ammonium sulphate, ammonium nitrate and urea were used, each at four different levels, *viz.*, 28 mg., 70 mg., 140 mg. and 210 mg. per 100 ml. of medium (levels 1–4 respectively). ANALAR chemicals were used throughout the investigation. Standard mycological technique (Rawlins, 1933) was followed. The pH of the media was adjusted to 5·5 prior to autoclaving, using a Beckman electronic pH meter and N/10 HCl or N/10 NaOH as reagents. The following determinations were made after 21 days' growth of the fungus in the cultures, these being incubated at room temperature (28–31° C.):—

1. dry weight of the fungal mat;
2. pH of the filtrate;
3. total nitrogen content of the fungal mat;
4. residual nitrogen in the filtrate:

   (*a*) total; (*b*) NO_3—N; (*c*) NH_4—N;

and 5. residual reducing sugars in the filtrate.

The above estimations were made using methods already described in detail elsewhere (Srinivasa Pai, 1953).

For assessing sporulation of the fungus, it was grown in the different media (6 replicates each) for 21 days in 10 ml. each of the medium in Pyrex test tubes. Spore counts were made using a Neuberg hæmacytometer. For statistical analysis spore counts per unit area were considered; spore numbers per ml. of the medium have been computed from these figures and they are represented in Fig. 1.

RESULTS

The results are presented in Table I and Fig. 1. The statistical analyses of some of the data are given in Table II.

From the results presented, the following conclusions may be drawn:

Changes in pH (Table I)

1. In media with ammonium sulphate, at all levels, the pH of the media was brought down to about 2 at the end of 21 days.

2. In media with potassium nitrate, ammonium nitrate and urea, at all levels, the pH was raised to about 6–7·2, except in the medium with urea level 1, where there was no change in pH.

Dry weight of fungal mat (Tables I and II, Fig. 1)

Statistical analysis of the data indicate that:

1. The highest mat weight was produced at potassium nitrate level 3, followed by ammonium nitrate levels 3 and 4.

TABLE I

Showing final pH, mat weight, N in mat, % N in mat, residual N and residual sugar in medium, spore counts per unit area and spore numbers per ml. of medium after 21 *days' growth of* Fusarium vasinfectum *in culture media supplied with different sources and levels of N*

No.	Final pH	Mat wt. in mg.	Nitrogen in mat mg.	% N in mat	Residual N in media			Residual sugar in gm.	Spore counts/ unit area	Spores per ml.
					NO_3–N	NH_4–N (in mg.)	Total			
1	6·7	418·7	16·38	3·9	4·14	3·60	8·25	..	675	10,800,000
2	6·75	514·4	20·43	3·9	37·32	6·48	44·46	..	612	9,792,000
3	7·0	714·2	32·19	4·5	100·20	2·86	102·50	..	586	9,376,000
4	7·2	421·4	17·03	4·0	175·00	4·23	183·30	..	710	12,860,000
5	2·1	211·1	19·69	9·3	..	9·05	9·38	0·25	217	3,472,000
6	2·0	241·1	23·30	9·6	..	44·94	45·50	0·44	121	1,936,000
7	2·0	181·1	17·28	9·5	..	118·20	120·20	0·74	170	2,936,000
8	2·1	164·4	16·67	10·1	..	189·48	191·90	1·06	200	3,136,000
9	5·5	445·5	26·30	5·9	..	1·86	1·86	..	416	6,656,000
10	6·5	418·6	31·99	7·6	..	5·12	31·40	0·17	685	10,960,000
11	6·9	413·0	30·76	7·4	..	5·94	108·90	0·19	900	14,400,000
12	7·1	424·0	41·14	9·7	..	5·98	168·50	0·40	653	10,448,000
13	6·9	423·6	13·65	3·2	3·16	8·48	12·86	..	252	4,016,000
14	7·0	516·7	17·36	3·4	14·28	34·20	51·86	..	262	4,192,000
15	7·1	602·8	22·23	3·6	47·94	68·98	112·84	..	137	2,112,000
16	7·1	627·3	24·54	3·9	84·10	98·40	182·40	..	108	1,728,000

Nos. 1– 4 : KNO_3 at 28, 70, 140 and 210 mg./100 ml. respectively;
5– 8 : $(NH_4)_2SO_4$ at 28, 70, 140 and 210 mg./100 ml. respectively;
9–12 : Urea at 28, 70, 140 and 210 mg./100 ml. respectively;
13–16 : NH_4NO_3 at 28, 70, 140 and 210 mg./100 ml. respectively.

Note : In analysing the data statistically (Table II), fractions of 0·5 and over have been taken as one; fractions less than 0·5 have been omitted.

2. The lowest mat weights were produced in the case of ammonium sulphate at all levels.

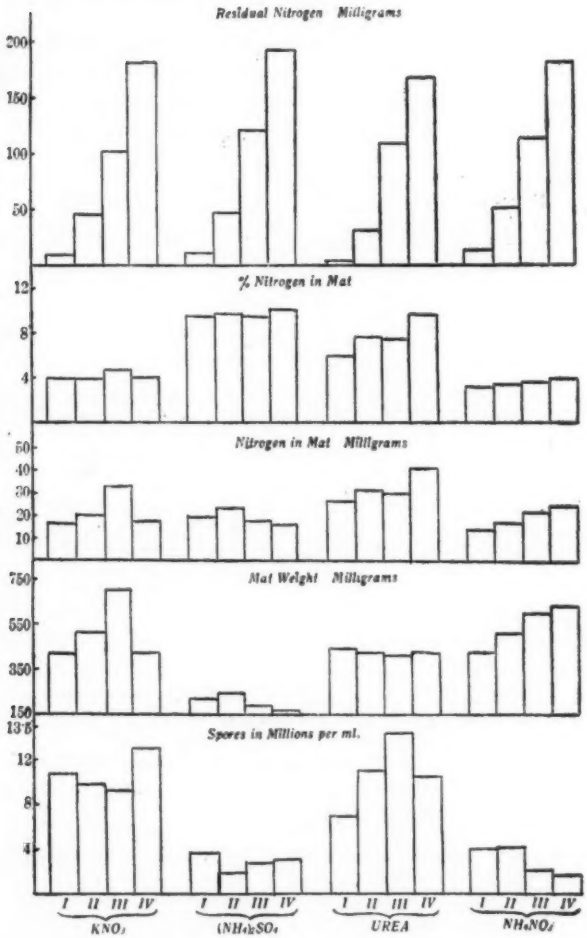
3. The differences between the mat weights in the other treatments were not significant.

Accumulation of nitrogen in fungal mat (Tables I and II, Fig. 1)

Statistical analysis of the data shows that:

1. Significantly the greatest amount of nitrogen was found in fungal mats raised in media with urea level 4 followed by potassium nitrate level 3 and urea levels 2 and 3.

2. The differences in the nitrogen content of the other treatments were not significant.



TEXT FIG. 1. Relation of nitrogen to growth and sporulation of *Fusarium vasinfectum* Atk.

Percentage nitrogen in fungal mat (Table I, Fig. 1)

The data show that:

1. The maximum percentage accumulation of nitrogen was in the case of ammonium sulphate at all levels and also in the case of urea level 4.

2. The minimum percentage accumulation of nitrogen was in the case of potassium nitrate and ammonium nitrate.

TABLE II

Showing statistical analyses of some of the data presented in Table I (the treatment numbers used are the same)

Mat weight :

Significance by F test : yes C.D. 1% : 8·4

Conclusion : 3, 16, 15, 2, 14, 9, 13, 12, 4, 1, 10, 11, 6, 5, 7, 8

Nitrogen in mat :

Significance by F test : yes C.D . 1% : 5

Conclusion : 12, 3, 10, 11, 9, 16, 6, 15, 2, 5, 4, 7, 8, 1, 13, 14

Total Residual Nitrogen (in Medium) :

Significance by F test : yes C.D. 1% : 4

Conclusion : 16, 4, 8, 12, 7, 15, 11, 3, 14, 6, 2, 10, 13, 5, 1, 9

Spores per unit area :

Significance by F test : yes C. D. : 7·5

Conclusion : 11, 4, 10, 1, 12, 2, 3, 9, 14, 13, 5, 8, 7, 15, 6, 16

3. Percentage accumulation of nitrogen was the same for a given salt, irrespective of the concentrations used, in the case of potassium nitrate, ammonium sulphate and ammonium nitrate. In the case of urea, however, there were increases in percentage accumulation of nitrogen in the fungal mat with increasing doses of the nitrogen source.

Total residual nitrogen in medium (Tables I and II, Fig. 1)

1. Residual nitrogen significantly increased from levels 1 to 4, in the case of all four sources, indicating that the greater the amount supplied the greater the amount left behind.

2. Comparing levels 1 and 2 for all four sources, it is found that significantly the lowest residual nitrogen was left behind in media with urea.

3. Comparing all four sources at level 3, it is found that significantly the greatest residual nitrogen was left behind in media with ammonium sulphate, while the lowest residual nitrogen was left behind in the medium with potassium nitrate.

4. Comparing all four sources at level 4, it is seen that the least residual nitrogen was left behind in the medium with urea; the differences between the other salts, *viz.*, ammonium nitrate, potassium nitrate and ammonium sulphate, were not statistically significant.

5. Almost complete depletion of nitrogen appeared to have taken place only in the case of the medium with urea level 1.

Residual reducing sugars in medium (Table I)

No residual sugar could be detected in media with all levels of potassium nitrate and ammonium nitrate, and urea level 1. In the other media different amounts of sugars could be detected after 21 days' fungal growth.

Sporulation (Tables I and II, Fig. 1)

1. Significantly the greatest sporulation occurred in the medium with urea level 3.

2. In media with ammonium nitrate and ammonium sulphate, at all levels, significantly lower number of spores was produced than in media with all levels of urea and potassium nitrate.

3. Comparing urea and potassium nitrate, it is seen that significantly the lowest sporulation occurred in the medium with urea level 1. Sporulation at other levels was not statistically different in media supplied with these two sources.

DISCUSSION

A comparison of the four different sources of nitrogen used in this investigation in relation to the growth, sporulation, etc., of *Fusarium vasinfectum* would be interesting.

The estimations of residual nitrogen at the end of 21 days' growth of the fungus show that there is probably an optimum amount of N required for growth of the fungus and additional supply of this element would not favour greater uptake of this element by the fungus, even if the C/N ratio is altered by varying the N content in the media. In other words, the utilization of N by the fungus is limited by certain factors, for instance the supply of carbon, and therefore increase in N in the medium without a proportionate or comparable increase in C would not encourage greater uptake of N. Inorganic salts of N (potassium nitrate, ammonium sulphate and ammonium nitrate) and also organic nitrogen (urea) had similar effects as far as uptake of N was concerned with increasing concentrations of the salts.

From the data presented in this paper, it is obvious that *F. vasinfectum* can utilize nitrates, ammonium salts and organic sources of N, and in so far as its ability to fix atmospheric N has not been demonstrated, it should be placed in Group II of the classification proposed by Robbins (1937).

The sporulation of the fungus and its growth, as indicated by its mat weight, differed with different sources and with different levels of these sources. Ammonium sulphate proved to be a poor source of N for vegetative growth and for sporulation of the fungus, although the percentage of N accumulated in the fungal mat was the highest when this salt was used.

The poor vegetative growth and sporulation of the fungus at all levels of ammonium sulphate is probably related to the very low final pH obtained in this case; it is also likely that the medium is made unfavourable for the growth of the fungus by the production of substances inhibiting its own growth. The high percentage of nitrogen contained in the mat raised in media with ammonium sulphate is attributable to the poor uptake and accumulation of substances other than N. Compared to ammonium sulphate, ammonium nitrate was a good enough source for vegetative growth, but not for sporulation. Nitrate (as potassium nitrate) and organic nitrogen (as urea) were both much better sources for vegetative growth as well as sporulation, and between them they appeared to be equally good in this respect, although in the medium with potassium nitrate level 3 significantly the greatest mat weight was produced and in the medium with urea level 3 there was significantly the maximum sporulation. Considering, however, the accumulation of N in the fungal mat, it is evident that there is a somewhat greater percentage of N in the mat in the case of urea than in the case of potassium nitrate; it is noteworthy that the high percentage N content in the mat in the case of urea is not the result of poor accumulation of substances other than N in the mat as in the case of ammonium sulphate. On the other hand, this situation is explained partly at least by the fact that there is greater uptake of this element in the case of urea, and hence also a greater depletion of N from the medium as shown by the data for residual N.

The data on residual sugar in the media after 21 days' growth of the fungus show the improbability of further growth of the fungus in the case of all levels of potassium nitrate and ammonium nitrate, and urea level 1, since there was no sugar left in the media. Just when complete depletion of sugar took place within the 21-day period is not clear from the present work since periodical estimations of residual nitrogen were not carried out. The earlier work of Srinivasa Pai (1953) indicates, however, that in the case of urea level 3, depletion of sugar from the medium would be complete after 18 days' growth of the fungus, and in the case of all levels of potassium nitrate at the end of 21 days. Srinivasa Pai did not include ammonium nitrate in his study. The presence of residual sugar in the case of all levels of ammonium sulphate and urea levels 2–4 indicates the possibility of further growth of the fungus; in any case, sugar is not a limiting factor for further growth.

Apart from the points mentioned above, no generalizations are possible, regarding the interrelationships of nitrogen uptake, nitrogen accumulation, sugar depletion, vegetative growth and sporulation of the fungus. Inverse correlation between vegetative growth and sporulation is often mentioned

in literature and, indeed, in the present work such a correlation is obvious in the case of ammonium nitrate where increasing doses of N increased vegetative growth but decreased sporulation; but this need not be a universal feature as is shown by the poor vegetative growth as well as poor sporulation when ammonium sulphate is used as the nitrogen source. Similarly, decrease in C/N ratio might increase the amount of N accumulated in the mat as in the case of ammonium nitrate (see Fig. 1); but this is related to the particular source of nitrogen used, since similar results are not obtained when the media are prepared with the same C/N ratios, but using different sources of N, like potassium nitrate, ammonium sulphate and urea.

It is admitted that the present work is of a preliminary nature. Only the effect of variations in nitrogen sources and levels has been studied, other conditions being kept constant. Variation in other factors, *e.g.*, the source and the amount of carbon supplied, would doubtless give a different picture, particularly of the relation of different sources and doses of N to the physiology of the fungus. The main purport of this paper is only to emphasize the complexity of the problem and the need for further detailed work. For, the application of inorganic and organic fertilizers with a view to enrich the nitrogen status of soils cannot but have its effects on soil-borne pathogens perennating in soils; these findings, moreover, may have implications in relation to host-fungus physiology as well, with which is linked up the vital question of host-resistance to soil-borne pathogens.

SUMMARY

The effect of four different nitrogen sources, *viz.*, potassium nitrate, ammonium sulphate, ammonium nitrate and urea, each at four different levels, *viz.*, 28 mg., 70 mg., 140 mg., and 210 mg. per 100 ml. of medium, on the growth, sporulation, nitrogen accumulation, and sugar and nitrogen depletion from media by *Fusarium vasinfectum* has been studied. The results are presented in detail and discussed. The main conclusions are summarised under " Results ".

ACKNOWLEDGEMENTS

We are grateful to Prof. T. S. Sadasivan for suggestions and criticisms during the course of the investigation, and to Sri. S. Suryanarayanan for the statistical analyses of the data.

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EXPERIMENTAL PNEUMOKONIOSIS CAUSED BY BERYL DUST IN GUINEA-PIGS

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Received April 18, 1952

(Communicated by Dr. K. V. Giri, F.A.SC.)

Pneumokoniosis is a generic term given to a group of pulmonary diseases caused by inhalation of certain dusts. All dusts are productive of functional disturbance, but those containing silica in the free state or in the form of fibrous silicate known as asbestos are capable of causing true pneumokoniosis. In all there is a fibrotic reaction to the irritant which slowly produces structural changes of the lung, resulting in marked disability and an increased susceptibility to secondary infection particularly to tubercle bacillus. These diseases result from prolonged exposure to the harmful dusts, in occupations such as mining, sandblasting, stone crushing and in the manufacture of refractories. Considerable hazard exists in preparing and mixing of powders in industry.

Movrogordato (1926–28) from his studies on the silicosis among the mine workers of Rand, came to the conclusion that most of the silicosis among the miners were infective from the beginning. He investigated the matter experimentally in guinea-pigs and rats which were exposed to phthisis-producing dusts followed by an infection, tuberculous or otherwise and found the lesions in the lungs far more fibriotic than in the control animals. But Carleton (1924) not being able to produce fibrosis while experimenting on guinea-pigs suggests that this observation may be due to the fact that guinea-pig's lung normally contains little fibrous tissue. His criterion for a dust to be harmful is its capacity to call forth plaque formation. Plaque formation according to him are conglomeration of thin squame-like cells, the cytoplasm of which is filled with dust particles. Haynes (1931) also states that plaque formation is never caused other than by potentially harmful dusts. In 1932, Kettle published with R. Hilton a paper on the technique of experimental pneumokoniosis. For the production of pulmonary lesions almost all workers previously had exposed animals for years in rooms or boxes in which a dusty atmosphere was maintained by some mechanical device. The inhalation method was expensive to maintain and a large proportion of the animals died before the experiment was completed. Kettle and Hilton

(1932) injected suspensions of dust in saline directly in to the trachea of rabbits and guinea-pigs. The mortality from this procedure was small and excellent results were obtained with a minimum of labour within a relatively short time. By this method they were able to test a large variety of dusts on a comparatively small number of animals, within a short time. Upto that time, it had been assumed by most workers that the injurious quality of silica depended on its hardness and insolubility, the damage being caused by mechanical action on the lung tissue. Guy and Kettle (1922) not only pointed out that silica is readily soluble in alkali and in the presence of living matter, but suggested that the deleterious action of silica depended on its solubility. Again in a paper published in 1934, Kettle showed that lesions can be rapidly produced in the lungs of guinea-pigs when a dust is combined with an infective process, whereas typical lesion can only be produced with great difficulty if at all by the dust alone. He held the view that while silicates by themselves may produce silicosis in the lungs of animals, that in man silicosis was normally always associated with tuberculosis, cases of simple silicosis being rare. W. R. Jones (1933) brought forward a strong case, in support of the view that sericite a potassium aluminium silicate—$K_2AlO_3.6SiO_2.2H_2O$—to be the cause of silicosis and that free silica (SiO_2) played a small part in the production of silicosis. He based his opinion on the birefringence studies of minerals obtained from the lungs of mine workers of South Wales Coal Fields, by 'sliming' the tissue with strong nitric acid and examining the mineral residue left after washing dissolved tissue under petrological microscope. He was led to believe that (1) the bulk of the mineral residue from silicotic lungs consists of sericite, (2) the number of quartz particles being less than the number of sericite particles, and (3) rocks containing small percentage of quartz but which contain fibrous silicates like sericite and silimanite gives off dust which has caused a large number of cases of silicosis in the collieries of anthracite district of South Wales Coal Fields. But this view has been criticised by Cooke (1935), Meller (1925) quoting S. Calderon states that the solubility of silica in distilled water to be 0·01 part per 100. The size of the grain has a remarkable effect on the solubility (smaller the size of the particle, the greater the solubility). McCrae (1913) had already emphasized that the particle size was of paramount importance in determining the chemical activity of the dust and physiological response to it. The chemical activity is of course increased in small particles because of the increased surface area. McCrae (1913) found in the lungs of mine workers of Transvaal Gold Mines, S. Africa, that 70% of the particles in silicotic lungs were less than 1 μ in diameter and that the largest was 10·5 μ in diameter. The average diameter

of lung alveoli in man being roughly around 250 μ to 300 μ in diameter (McDowell, 1939) and there is no reason why larger particles should not enter them. In fact it has been found to be so.

Under industrial conditions workers are exposed to mixed dusts and it is difficult to be sure of the harmful agent. A critical study of the problem of silicosis studied by various workers in this field also shows that they have used a variety of dusts and of mixed composition. Hence the evaluation to any definable chemical or physico-chemical state of the silicate structure is rendered difficult. This investigation was undertaken to throw light on the nature of tissue reaction to beryl as detailed structural studies made on this mineral were available (Bragg, 1937; Wells, 1945) and although the toxicological hazards of beryllium as the oxide and metal dust are reported in literature [Machle, Beyer and Tebrock (1949), Eisenbud *et al.* (1949), and Aub and Grier, (1949)], the study on the ore is lacking.

MATERIALS AND METHODS

Guinea-pigs of average weight of 250 gm. were chosen as the experimental animals and they were kept separately in individual cages. Food and water were made available in sufficient amounts to keep the animals healthy. Twelve animals were used for the experiment so that they could be sacrificed at various intervals. Beryl dust of mesh −325 (particle size below 44 μ), was administered to the experimental animals. Usually about 1·5–2 gm. was suspended in normal saline and this amount of the dust was administered intratracheally, spreading over several days so that each day the animal received only a fraction of the total dosage. The method of administration was a slight modification of the technique adopted by Kettle (1934). The animal was held firmly by an assistant and a hypodermic syringe with an attached rubber catheter filled with the dust suspension was pushed back towards the posterior nares and the suspension was slowly ejected in to the trachea. The animals received a single dose on each day till the required dose was over. The animals experienced violent coughing and choking sensation but very soon recovered from the effect.

Histology.—The animals were killed at varying intervals and the various tissues like lungs, liver, spleen, kidney and suprarenal were dissected out and fixed in Bouin's fluid as a routine and some were fixed in Zenker-Formol for comparison. Paraffin sections were stained as a routine with Delafield's Hæmatoxylin and Eosin. Weigert's iron hæmatoxylin and Van Gieson's stain were used to display fibrous tissue.

Hæmatology.—Blood smears at varying intervals were made and stained with Leishmann stain for making differential count of leucocytes.

Total red cell count, leucocyte count, and hæmoglobin estimations were done on the same day.

Experimental

The experimental animals survived the aspiration of the dust solution, except for a few minutes of respiratory embarrassment and continued to live for varying periods. The maximal survival period was 150 days after the start of the experiment. The animals appeared sick after the 60th day and showed only signs of respiratory difficulty.

The animals were killed and tissues taken for histological study at three intervals. The first being on the 15th day, and the other two intervals being 60th and 100–150th day. The animals dying during the course of the experiment were examined for any post-mortem changes and organs taken for histological examination. Post-mortem examination of the lungs and other organs like liver, spleen and the kidney showed no profound differences between the lesions which became apparent only on microscopic examination. There was naturally a good deal of variation in the extent to which tissue damage had progressed in different cases of approximately equal duration. The sequence of events in the damaged material, so far as the present investigations show, appear to be as follows and are illustrated in the microphotographs (Plates VIII and IX).

Microscopic Appearances

Lungs.—Histopathological changes in the lungs showed on the 15th day evidence of even distribution of the dust in the alveoli and slight thickening of the alveolar septum. Inflammatory changes with cell infiltration into the alveoli were not met with. A certain amount of large particles, of size 15 μ, were seen in the alveoli without causing any fibrous reaction. As the disease progressed the consolidation of lungs became marked with thickening of the alveolar walls causing pronounced and lasting reaction. Plaque formation, the local aggregation of the dust laden phagocytes did not occur to any extent. The characteristic silicotic nodule was not observed even in the last stages, but lobular emphysema was seen particularly at the apices and bases before the animals died (Plate VIII).

Liver and Spleen.—The liver showed a series of changes varying from patchy hydropic degeneration of liver cells and slight fatty changes to central or midzonal necrosis. There was nothing characteristic about the pathology of the damaged liver cells. There was no sign of hæmorrhage or pigment deposition. Spleen and tracheobronchal lymph nodes showed no marked changes (Plate IX, Fig. 1).

Kidneys.—The kidneys showed constant and relatively slight changes confined to the tubular epithelium. These cells appearing filled with fine granules were often swollen and some times vacuolated. Glomeruli were not affected (Plate IX, Figs. 3 and 4).

Suprarenal.—Adrenal cortex showed hypertrophy and the cortical cells showed extensive foamy degeneration. At first the cortical degeneration was confined to zona glomerulosa and later it was coextensive with zona fasciculata. There was no evidence of cortical necrosis or hæmorrhage (Plate IX, Fig. 2).

Hæmatological studies revealed a hypochromic macrocytic anæmia in acute conditions. But the total leucocyte count remained unaffected. The differential count of leucocytes on the other hand showed lymphocytosis.

DISCUSSION

The results recorded in this paper are a part of a wider investigation in an attempt to correlate the structural chemistry of the dust in relation to its activity towards tissues in producing reaction.

Beryl occurs in nature as beryllium aluminium silicate, $3BeO, Al_2O_3 \cdot 6SiO_2$. The structure of beryl can be seen from Fig. 1. The cyclic

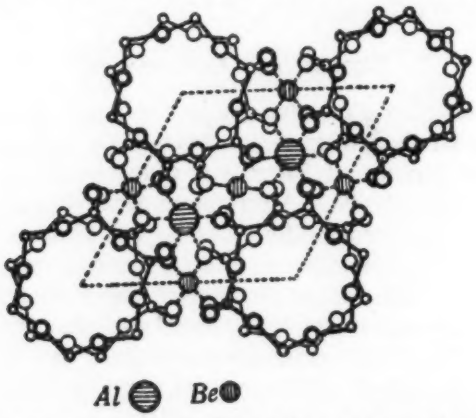

FIG. 1. Plan of the structure of beryl, $Be_3Al_2Si_6O_{18}$. "Atoms belonging to complex ions lying at different heights above the plane of the paper are shown as heavy and light circles to show the nature of the co-ordination groups around the metal ions. Many oxygen atoms are omitted for the sake of clarity" (After Wells).

ions ($Si_6O_{18}^{12-}$) are arranged in sheets with their planes parallel, but the structures are not layer structures, as they appear in the plan, as the metal ions lie between the sheets and bind together the rings in different sheets

In the case of beryl the Be^{2+} ions are in tetrahedral and the Al^{3+} in octahedral holes (Wells, 1945). In these positions they link the oxygen atoms of each sheet of rings to the oxygen atoms of the sheets above and below. The whole structure is thus bound together laterally and vertically (Bragg, 1937).

The pathogenicity of silica in relation to silicosis is thought by many people to depend on its solubility (Gye and Kettle, 1922; Cooke, 1935). Mills (1931) also has furnished proof that silica is soluble in animal tissues, finding that the silicious spicules of fresh-water sponge are slowly dissolved when introduced into the tissues of dogs, rabbits and guinea-pigs. If the harmful effects of silica in the cells are related to its dissolution, then it is expected that silica particles in the lung would dissolve in the fluids and such a dissolution of silica in the lung should result in the passing of silicic acid into the blood followed by its excretion into the urine. The evidence for this is conflicting. Inhalation of crystalline silica has led to minor changes only (King *et al.*, 1938). Fresh fracture has been suggested as cause of rapid action. Beyersdorfer (1948) has explained the effect on the crystalline structure of quartz of a shattering force liberating many tetrahedra with a free valency and a greater chemical activity. But silicosis has been caused in man and in experimental animals by quartz and flint long after they have been ground (Middleton, 1936; Belt, 1939; and Charles *et al.*, 1948). Tissue changes produced in the lung may be and often are of a complex nature. But the unit of lesion is a silicotic nodule. In our experiments, there were no evidence of a fibrous reaction even though the dust remained in the lung for a long time. Plaque formation, that is to say, the incipient silicotic nodule formation was also not observed. It seems probable that beryl by itself lacks the basic physico-chemical properties required to produce fibrosis. It seems that typical lesion of the phenomenon of silicosis is produced only when an accessory factor, *viz.*, infection, tubercular or otherwise, is present (Kettle, 1934).

The size of the dust particles also appears to be of considerable importance. The size of the particles causing silicosis has been generally regarded as being of the size 1–3 μ according to Sayers and Jones (1938) but in fact no lower agreed limit of size has been fixed. In our series of experiments, the largest sized particle was 15 μ in the alveoli though a much bigger particle can be accommodated as the lung alveoli of guinea-pig is on the average 32–75 μ in diameter in normal animals.

The symptoms of beryllium poisoning among workers are anorexia marked prostration and loss of weight. These symptoms must be due to the fact that the adrenal cortex has sustained severe damage. Early damage

is seen in the zona glomerulosa of the adrenal gland of the guinea-pig which is the seat of harmones influencing electrolyte balance, while zona fasciculata is being affected gradually. Deane and Greep (1946) have shown that zona fasciculata secretes corticosterone-like harmones which govern gluconeogenesis. The cortical hypertrophy in the early stages suggests an increased secretion of cortical harmone and latter its lipid depletion indicates an output of the harmone below the physiological requirements of the animal.

SUMMARY

1. The damaging effects of inhalation of beryl dust on guinea-pigs lung and other organs are studied using Hilton and Kettle technique.

2. It has been shown that beryl produces in the lungs chemical pneumonitis at the onset and later leads to marked atrophic emphysema. No evidence of fibriosis or silicious nodule formation was noted.

3. Other organs like liver and kidney showed signs of damage, but adrenal cortex showed definite pathological changes in the zona glomerulosa and zona fasciculata with the duration of the experiment.

4. The importance of concurrent study of the morphological chemistry of the dust in relation to its role in the production of silicosis is emphasised as such a method if followed by workers in this field may render a rational approach to the fundamental problem of silicosis.

ACKNOWLEDGEMENTS

Authors thanks are due to Prof. K. V. Giri for his keen interest and encouragement in the course of the work and to Drs. N. N. De and K. P. Menon for helpful suggestions and criticisms on the work.

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VIDEO

Proc. Ind. Acad. Sci., B, vol. XXXVII, Pl. VIII

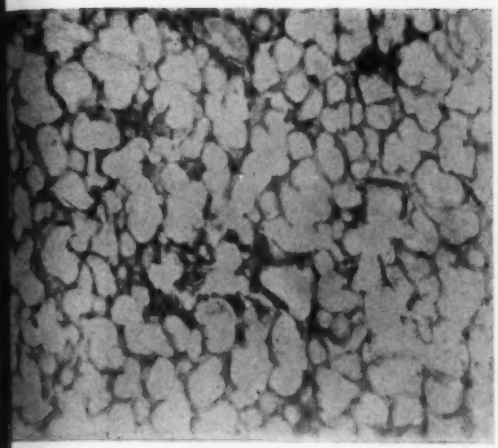
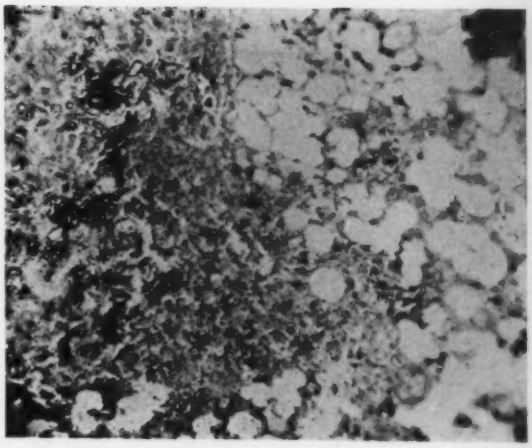


FIG. 1



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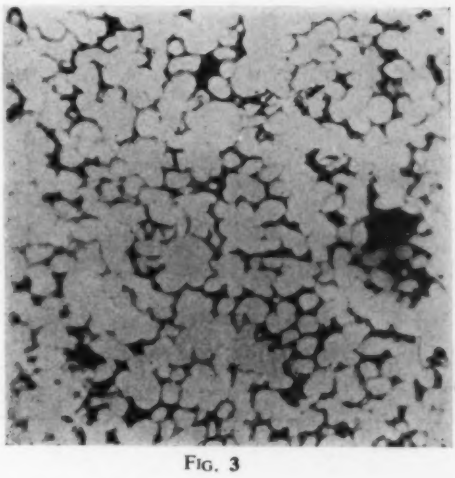

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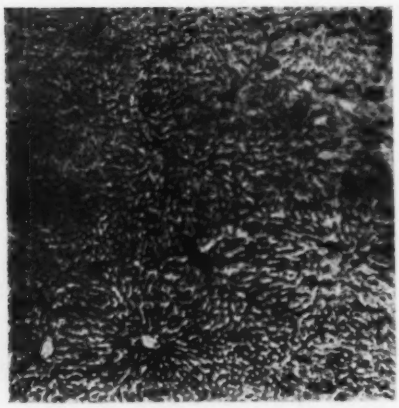

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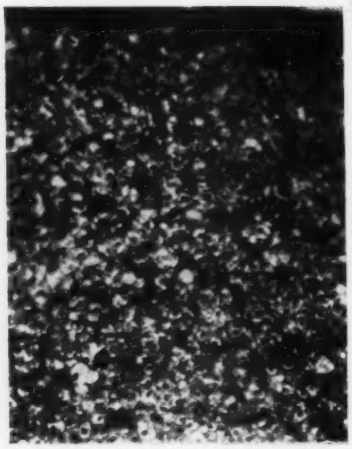

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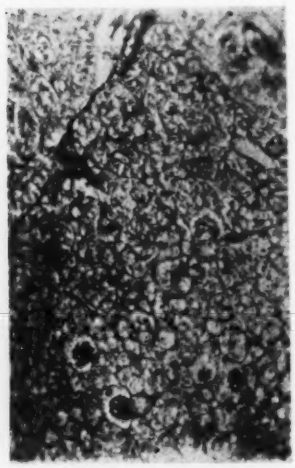

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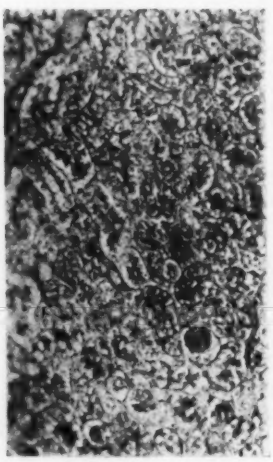

FIG. 4

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Explanation of Plates

Plate VIII

Experimental Pneumokoniosis

Figs. 1–3. Fig. 1. Section of normal lung of guinea-pig. Note the extreme scantiness of connective tissue except around the terminal bronchi. H & E, × 60. Fig. 2. Section of lung of guinea-pig killed 65 days after intratracheal injection of a suspension of beryl dust. The lung is solid with cells containing dust. Note absence of fibrosis, the adjacent alveolar sacs are dilated. Particles of beryl also can be seen in the lung tissue. H & E, ×60. Fig. 3. Section of lung of guinea-pig killed after 100 days intratracheal injection of beryl dust. Atrophic emphysema with breaking down of alveolar walls. Alveolar sacs are not dilated. H & E, ×60.

Plate IX

Experimental Pneumokoniosis

Figs. 1–4. Fig. 1. Liver of guinea-pig exposed to beryl dust. 60th day. Midzonal necrosis. The cells around the central veins of the lobules and midzonal region are affected. H & E, × 60. Fig. 2. Suprarenal of guinea-pig exposed to beryl dust. 60th day. Zona glomerulosa and zona fasciculata show evidence of degeneration and few cells are enlarged probably due to compensatory hypertrophy. H & E, × 100. Figs. 3-4. Kidney of guinea-pig exposed to beryl dust. 60th day. Shows marked swelling of tubule cells and occlusion of lumina. Glomeruli are not affected. H & E, × 60.

395–53 Printed at The Bangalore Press, Bangalore City, by C. Vasudeva Rao, Superintendent
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ERRATA

Vol. XXXVII, B, No. 3, March 1953

"Fungi Imperfecti from Madras—IV," by C. V. Subramanian

P. 104, line 2 should *read*: "Setæ simplices, haud ramosæ, septatæ. Conidiophori simplices, apice globose fertili qui cupulatus".

P. 104, line 30 should *read*: "with setæ. Setæ simple, unbranched, septate. Conidiophores simple, with globose fertile apex".

ERRATA

Vol. XXXVII, No. 1, March 1953

"Rungi Imperfecti from Madras—IV," by C. V. Subramanian

P. 104, line 2 should read: "Sete simplices, basi ramosae, separatae.
Conidiophori simplices, apice globose fertili qui cupulatus".

P. 104, line 30 should read: "with sete. Sete simple, unbranched,
septate. Conidiophores simple, with globose fertile apex".

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